



## Controlled and Scalable Differentiation of Human Pluripotent Stem Cells into Radial Glia and Astrocytes Bypasses Neurogenesis

Vukasin M. Jovanovic<sup>1</sup>, Claire Malley<sup>1</sup>, Carlos A. Tristan<sup>1</sup>, Elena Barnaeva<sup>1</sup>, Pei-Hsuan Chu<sup>1</sup>, Jennifer Colon Mercado<sup>2</sup>, Michael E. Ward<sup>2</sup>, Anton Simeonov<sup>1</sup>, Ilyas Singeç<sup>1</sup>

<sup>1</sup> National Center for Advancing Translational Sciences (NCATS), Division of Preclinical Innovation, Stem Cell Translation Laboratory (SCTL), Rockville, MD 20850, USA

<sup>2</sup> National Institute of Neurological Disorders and Stroke (NINDS), Inherited Neurodegenerative Disease Unit, Bethesda, MD 20892, USA National Institutes of Health (NIH)

## Introduction

Brain development starts with the formation of the neural tube followed by the emergence of radial glial (RG) cells that act as neural stem cells (NSCs) and sequentially produce neurons and then glial cells at later stages. Astrocytes are considered key players in normal brain function and during pathogenesis of various neurological and psychiatric diseases. However, the molecular mechanisms that control astrogliogenesis during development remain poorly understood. Hence, derivation of astrocytes from human pluripotent stem cells (hPSCs) has been inefficient, variable, and long-lasting. Current methods require extended periods of cell culture (6 months and longer), use of xenogenic material, and forced expression of transcription factors. Here, we developed a highly efficient chemically defined astrocyte differentiation strategy that requires neither animal serum nor genetic manipulation. Surprisingly, our in vitro approach bypasses neurogenesis, which is known to always precede astrogliogenesis similar to in vivo brain development. Specifically, by modulating gliogenic cell signaling pathways, we first differentiated hPSCs into FABP7+ RG-like cells in 7 days. These cells were then stepwise differentiated into astrocytes with over 95% efficiency in less than 30 days. Extensive molecular and functional characterizations (e.g. single cell analysis, calcium imaging, co-culture experiments), cell grafting into mouse brains, and disease modeling (e.g. Alexander disease) confirmed the specific identity and translational utility of hPSC-derived astrocytes. Lastly, our method was automated using a robotic cell culture system, which enabled the standardized production of billions of human astrocytes. Collectively, our results shed new light on cell fate specification by identifying remarkable plasticity in neural lineage progression that can be exploited to engineer hPSC-derived astrocytes for high-throughput drug screening and regenerative medicine.

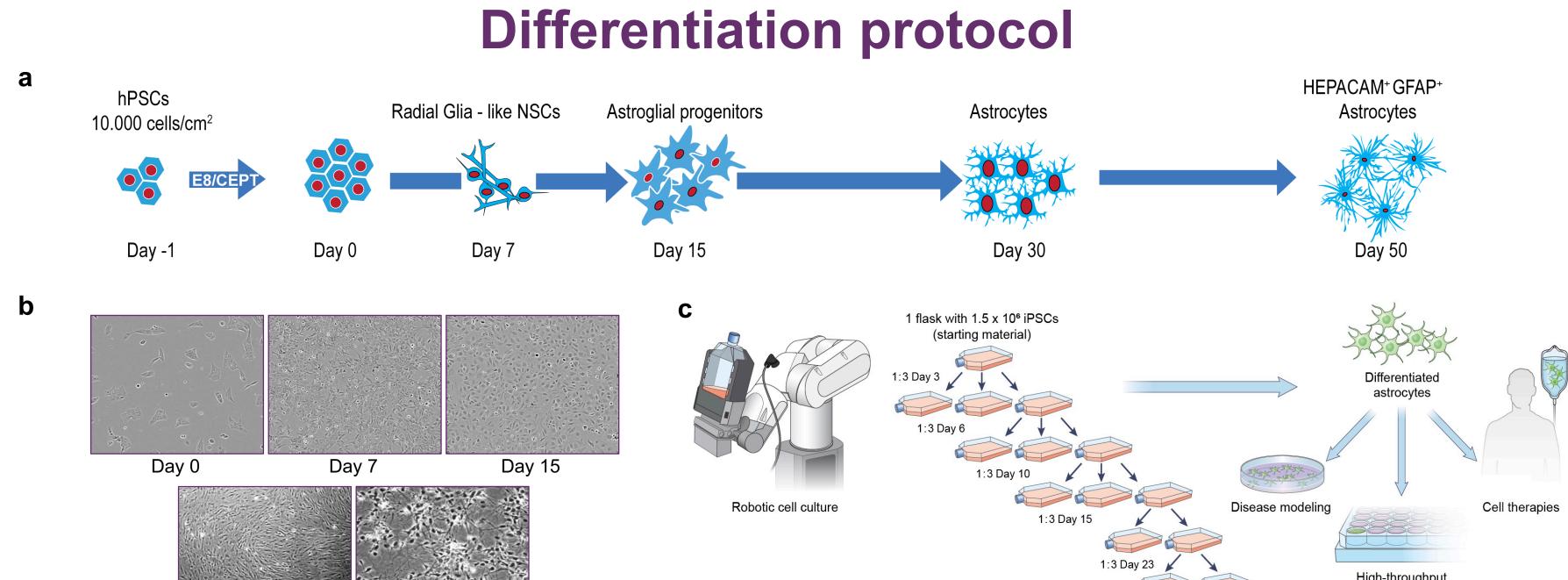
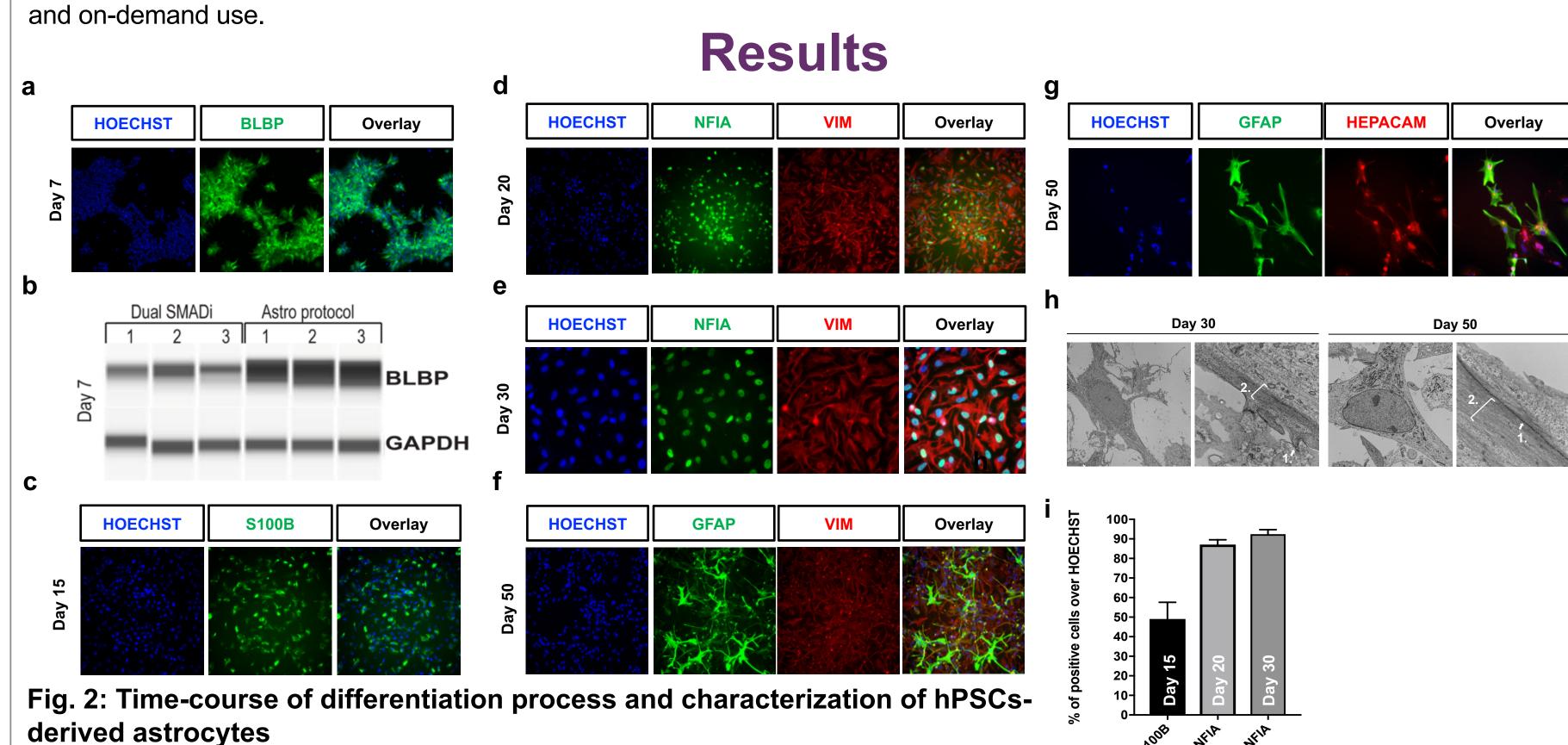


Fig. 1: Automated and scalable astrocyte differentiation from human pluripotent stem cells.

Day 50

- a. Novel approach for stepwise and controlled differentiation of hPSCs into radial glia-like cells and astrocytes
- **b.** Phase-contrast images depict different developmental stages (Day 0 = pluripotent; Day 30 & 50 = immature & mature astrocytes). c. Differentiation protocol is amenable for automated cell culture and production of billions of human astrocytes for cryopreservation



a. Immunostainings show efficient differentiation of hPSCs into BLBP+ radial glia-like cells in a week. b. Western blot analysis at day 7 reveals that radial glia marker BLBP is expressed at much higher levels when using this new protocol versus the dual-SMAD inhibition method (d-SMADi). Numbers 1-3 indicate technical replicates for each condition. c. Expression of S100B<sup>+</sup> in astroglia progenitors at decomposition decomposition. day 15. d, e. Glial markers NFIA and VIMENTIN are strongly expressed at day 20 and day 30. f, g. Culturing day-30 astrocytes for additional 20 days in serum-free conditions results in expression of more mature glial markers (GFAP, HEPACAM). h. Ultrastructural analysis of iPSC-astrocytes at days 30 and 50 reveals well-developed cell junctions (1.) and intermediate filament structures that are typical of astroglial cells (2.) i. Quantification of cells expressing S100B (day 15) and NFIA (days 20 & 30) show efficient differentiation and purity of astroglial cultures.

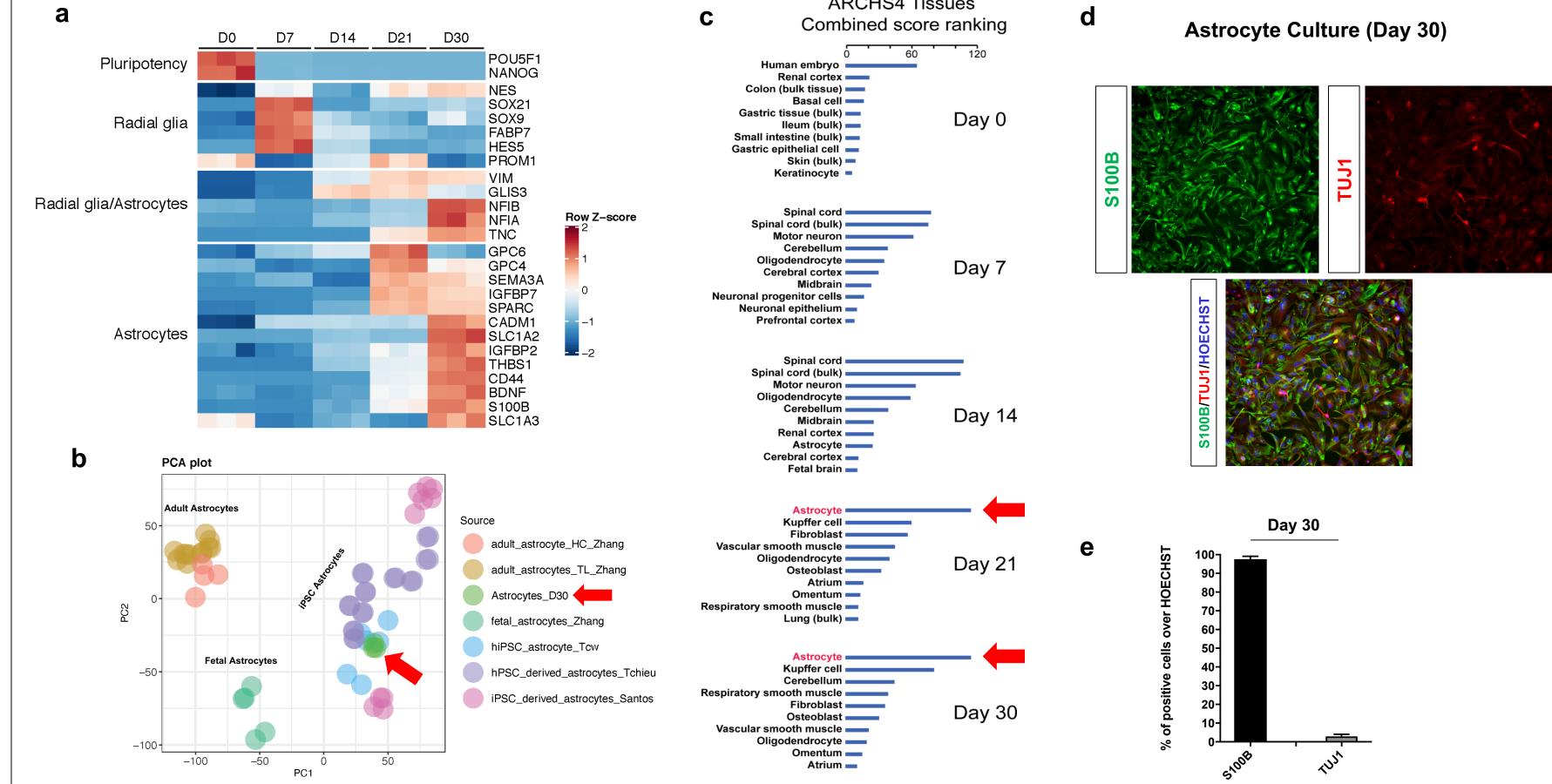


Fig. 3: Characterization of hPSCs differentiating into astrocytes

a. Heat-map (RNA-seq) illustrating expressed genes by pluripotent cells, radial glia, and astrocytes (day 0-30). b. SRA comparison of transcriptomes of iPSC-astrocytes (day 30, present study, red arrow) versus previously published iPSC-derived astrocytes (TCW et al. 2017; Santos et al. 2017; Tchieu et al. 2019) and their in vivo counterparts (Zhang et al., 2016). c. EnrichR analysis (Lachmann et al., 2018) comparing the transcriptomes of differentiating iPSCs at different timepoints (day 0-30). The gene expression signature of iPSCderived astrocytes is congruent with their in vivo counterpart (red arrows). d. Expression of S100B and TUJ1 at day 30. e. Note the very low percentage of TUJ1<sup>+</sup> neuronal cells, whereas the vast majority of cells express the glial marker S100B.

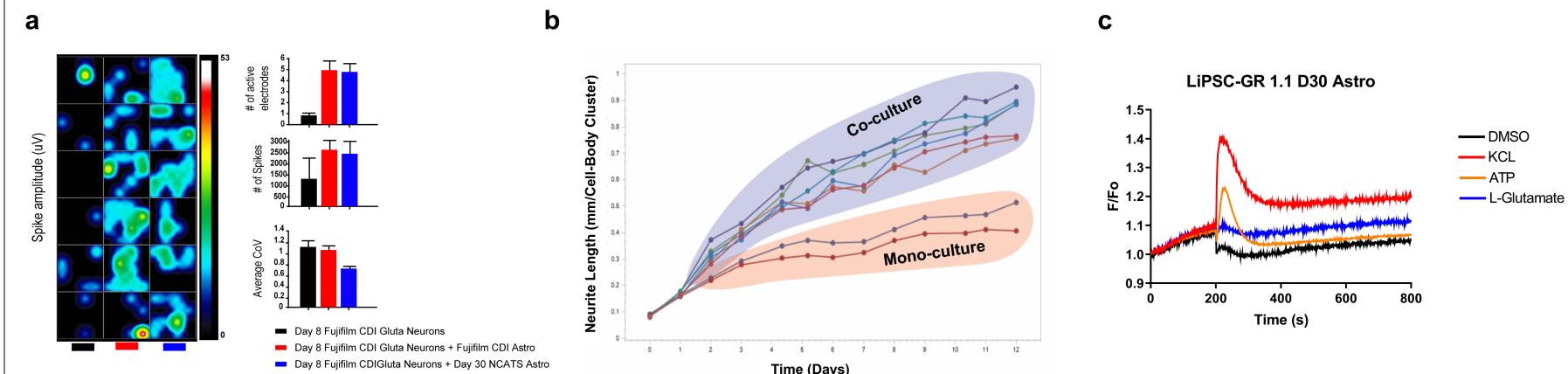
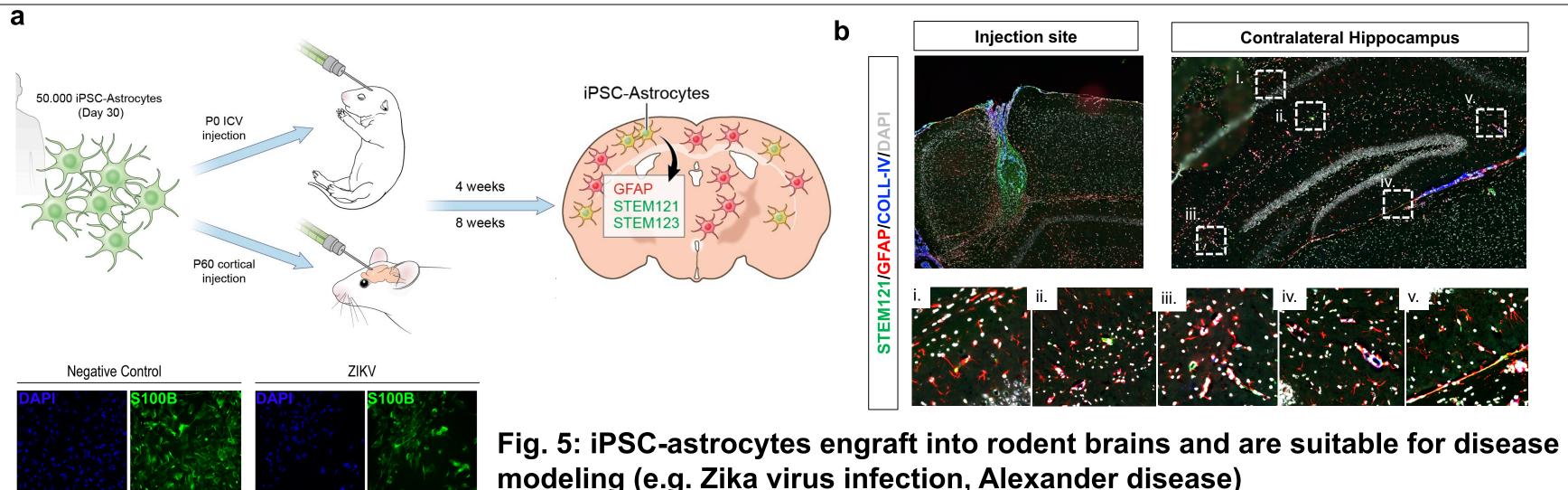


Fig. 4: Functional analysis of iPSC-derived astrocytes

a. Glutamatergic neurons (FUJIFILM CDI) show increased number of spikes when co-cultured with control astrocytes (red; FUJIFILM CDI) or NCATS astrocytes (blue) for 8 days as measured by multi-electrode arrays. **b.** IncuCyte live-cell imaging of i3-Neurons, stably expressing cytosolic mScarlet to label neurites (Tian et al., 2019), document increased neurite outgrowth when co-cultured with NCATS astrocytes. c. Day 30 iPSC-astrocytes display calcium transients in response to appropriate stimuli.



modeling (e.g. Zika virus infection, Alexander disease)

a. Illustration of cell grafting experiments performed in newborn and adult mice. b. Confocal image shows that the cortical injection site is marked by strong immunoreactivity for STEM121 (human cytoplasm marker) and depict numerous human cells that have migrated to the contralateral hemisphere. Example images taken from the hippocampus (boxed areas shown at higher magnification). Note the numerous cells double-stained for GFAP and STEM121. c. Day 30 iPSC-astrocytes can be infected with ZIKA virus. d. Day 50 iPSCastrocytes derived from an Alexander disease (AxD) patient show perinuclear GFAP-positive aggregates (yellow arrowheads).

Funding Source: NIH Common Fund; NCATS Intramural Research